Bisphenol A Induces a Profile of Tumor Aggressiveness in High-Risk Cells from Breast Cancer Patients

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Abstract

Breast cancer outcome is highly variable. Whether inadvertent exposure to environmental xenobiotics evokes a biological response promoting cancer aggressiveness and a higher probability of tumor recurrence remains unknown. To determine specific molecular alterations which arise in high-risk breast tissue in the presence of the ubiquitous xenoestrogen, bisphenol A (BPA), we used nonmalignant random periareolar fine-needle aspirates in a novel functional assay. Early events induced by BPA in epithelial-stromal cocultures derived from the contralateral tissue of patients with breast cancer included gene expression patterns which facilitate apoptosis evasion, endurance of microenvironmental stress, and cell cycle deregulation without a detectable increase in cell numbers. This BPA response profile was significantly associated with breast tumors characterized by high histologic grade ($P < 0.001$) and large tumor size ($P = 0.002$), resulting in decreased recurrence-free patient survival ($P < 0.001$). Our assays show a biological "fingerprint" of probable prior exposure to endocrine-disrupting agents, and suggest a scenario in which their presence in the microenvironmental milieu of high-risk breast tissue could play a deterministic role in establishing and maintaining tumor aggressiveness and poor patient outcome. [Cancer Res 2008;68(7):2076–80]

Introduction

The challenges encountered in studies of breast cancer and the environment include a lack of reliable exposure and risk assessment tools. Issues related to individual differences in breast cancer susceptibility pose critical difficulties in the experimental design of such studies. Despite these obstacles, epidemiologic evidence suggests a strong association between breast cancer and prolonged estrogen exposure (1). This raises concerns regarding the increasing prevalence of xenoestrogens, such as bisphenol A (BPA), in the environment, leading to a higher exposure burden over time.

BPA is widely found in polycarbonate plastics, which enter human consumption by leaching out from food and beverage containers, and in epoxy resins used as dental sealants (2, 3). It mimics endogenous estrogen and has a reasonably high affinity for estrogen receptors (ER), thereby eliciting profound effects, including the development of preneoplastic mammary lesions in rodents within a few months of exposure (4). Although reports on cancer cells themselves have described the growth-stimulating effects of several xenoestrogens, particularly in estrogen-dependent breast cancer cell lines (5), the direct effects of such exposure on cancer-prone human breast tissue and its role in further malignant development and behavior has not been fully addressed by such studies. To capture early changes induced by continuous BPA exposure of susceptible breast tissue, we have evaluated its effects on global gene expression in clinical samples of random periareolar fine-needle aspirates (RPFNA) propagated in an *in vitro* setting in which epithelial-stromal feedback is facilitated as this is reportedly a more sensitive approach for the detection of both direct and indirect estrogenic effects (6). To test the hypothesis that the biological response of high-risk cell cultures to BPA recapitulates the path followed by tumor cells in the clinical tissue of similarly exposed individuals, a search was conducted for subsets of breast cancer which mirrored the gene expression changes induced *in vitro*. Confirmed demonstrations of such a parallel compel serious consideration of the consequences of BPA exposure in susceptible individuals.

Materials and Methods

Cell culture and hormone exposure. Using a minimally invasive procedure, nonmalignant RPFNAs were collected from unafflicted, contralateral breast tissue of patients at the time of surgical resection of the primary breast lesion with written informed consent and institutional review board approval. Nonmalignant samples derived from eight patients receiving surgical treatment only were used in the experiments described here. Patients were diagnosed with invasive ductal carcinoma (four cases: ages 43, 52, 66, and 79 years), ductal carcinoma *in situ* (one case: age 47 years), atypical intraductal papilloma (one case: age 41 years), and fibroadenoma (two cases: age 47 years).

After 2 to 3 weeks of propagation in optimized growth medium (7), cells were cocultured in 0.4 μm inserts with hanging geometry (Becton Dickinson) with nonmalignant breast tissue fibroblasts at a 3:1 ratio. Continuous 7-day treatments consisted of luteal phase concentrations of 17-β-estradiol (E2, Sigma) and progesterone (PG, Sigma), and 10^{-7} mol/L of BPA (Sigma). For immunofluorescence, cell monolayers fixed with 1:1 acetone/methanol were tested for reactivity with mouse monoclonal pan-cytokeratin (34BE12) and vimentin (AMF17b) primary antibodies, in conjunction with Alexa 488–conjugated antimouse (Invitrogen), and evaluated using confocal microscopy.

Gene expression analysis. Epithelial cell RNA derived from duplicate wells of each treatment using the RNeasy kit (Qiagen) was labeled and hybridized to Human Genome U133 Plus 2.0 Arrays (Affymetrix) and genome-wide expression profiles were generated as previously described (8). Data from the CEL files of 59 independent arrays was normalized using dChip, and the expression level was modeled using the perfect match–only model.

Gene set analysis was used to assess the significance of predefined gene sets (9), which identified differentially activated or repressed genes in one of

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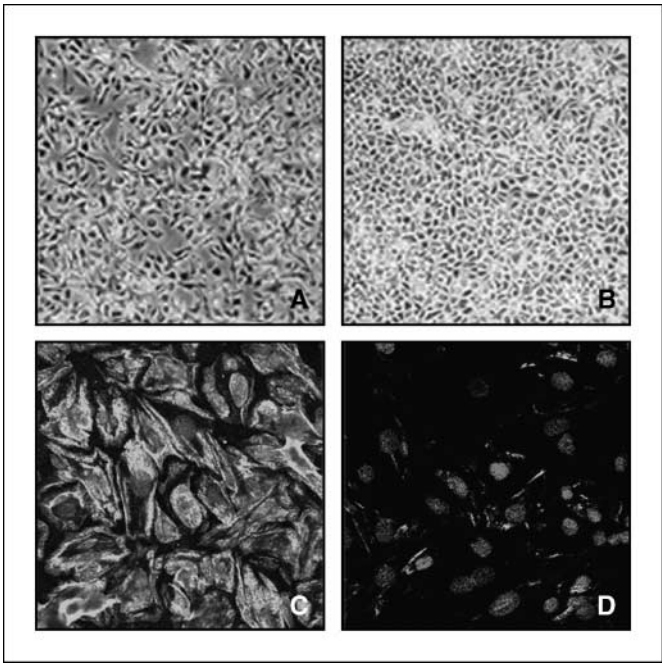

Figure 1. Authentication of the epithelial phenotype of high-risk RPFNA samples (*A* and *B*). Bright-field microscopic images of 2-week-old primary epithelial cultures from two independent cases. Immunolocalization of cytokeratin (*C*) and vimentin (*D*). Note the strong expression of the cytoplasmic cytokeratin network only. Nuclei were counterstained with propidium iodide.

the two following sample groups comparing no-treatment versus E2, E2 versus E2 + PG, and E2 + PG versus E2 + PG + BPA. The C2 functional gene sets of Subramanian et al. (10) were evaluated based on a two-class paired test using significance analysis of microarrays (11). Significant gene sets were chosen from 100 permutation tests at <5% estimated false discovery rate (FDR). Within each gene set, some genes were activated whereas others were repressed. A breast cancer classifier was derived for each treatment by using all activated genes in activated gene sets and repressed genes in repressed gene sets.

Class prediction. The classifier trained on the expression profiles of E2, PG, and BPA-exposed cell cultures was applied to breast cancer array data publicly available via the National Center for Biotechnology Information Gene Expression Omnibus. Data sets GSE5460 and GSE6532 were selected for matching the Affymetrix platform which we used, and the availability of patient follow-up and/or clinicopathologic information on >100 patients. The training and test sets were first normalized so that the within-set average was 0 and within-set SD was 1 for the expression values derived as follows:

$$x\prime_{ij} = \frac{x_{ij} - \bar{x}_i}{\sqrt{\mathrm{var}(x_i)}}$$

$\bar{x}_i$ and var(x_i) is average and variation of expression values of gene i along the samples within a set. We calculated the feature w_{kj} of gene set S_k of sample j by taking average expression values of associated genes (selected as described above) to the gene set k. n_k is the number of genes associated with the gene set S_k.

$$w_{kj} = \frac{1}{n_k} \sum_{I \in S_k} x\prime_{ij}$$

The nearest shrunken centroid classifier was used to train and classify test samples. This algorithm estimated the class probabilities by analogy to Gaussian linear discriminant analysis. The likelihood score of a tested sample was defined as the ratio of the probability of the predicted class to the probability of the opposite class. Comparisons for tumor size and grade were done for all samples of GSE5460 and GSE6532. ER status variation (available only for GSE5460) and patient outcome (available only for GSE6532) were evaluated in samples with likelihood scores >2. P values were determined by Fisher test.

Results and Discussion

RPFNA is a simple procedure, resulting in cells with optimal cytomorphologic evaluability (reviewed in ref. 12). By optimizing *in vitro* propagation, the limitations of low cell yield, particularly of samples derived from nonmalignant cancer-prone tissue, were circumvented. An experimental setup comprised of RPFNA-derived pure epithelial cells (Fig. 1), in coculture with breast fibroblasts, enabled cross-exposure to secreted gene products in the presence of physiologic hormone levels. At these concentrations, E2, as well as endocrine-disrupting agents are known to induce proliferation in established breast cancer cell lines. However, in our nonmalignant cocultures, cell numbers were consistent for all treatments relative to control for the entire experimental duration.

Global gene expression analysis of the high-risk epithelial component of the cocultures identified 11, 37, and 38 differentially expressed gene sets in gene set analysis–based comparisons of control versus E2, E2 versus E2 + PG, and E2 + PG versus E2 + PG + BPA expression profiles, respectively. Most significantly, PG and BPA exposure induced dramatically opposing patterns of expression for six independent gene sets in these cells. The combined pattern of expression of these six sets, designated as the cancer-prone response profile (CPRP), depicts a distinctive biological response of high-risk breast epithelial cells to E2, PG, or BPA (Fig. 2). These gene sets are curated in the Molecular Signatures Database C2 functional collection[3] as: IGF_VS_PDGF_UP (73 genes), IGF1_MTOR (20 genes), MTOR (23 genes), FERRANDO_MLL_T_ALL_DN (87 genes), BLEO_MOUSE_LYMPH_HIGH_24HRS_DN (34 genes), and CANTHARIDIN_DN (52 genes).

Consistent with previous reports in breast cancer cell lines (13, 14), the overall transcriptional profiles of E2 and BPA treatment were strikingly similar in primary cultures of nonmalignant breast epithelial cells as well. Within the six gene sets, E2, PG, and BPA response was distinctive for 28, 123, and 52 genes, respectively (Fig. 3). The distinctive E2-activated CPRP genes were associated with stress response, glucose metabolism, antiapoptosis, cell cycle regulation, and DNA replication and repair. Although these were significantly repressed in epithelial cells exposed to PG, the genes in pathways associated with cell differentiation, muscle development and contraction, and motility were significantly activated. Notably, the addition of BPA to growth medium supplemented with the other two hormones resulted in a dramatic reversal of PG-induced effects. Thus, in the presence of BPA, nonmalignant epithelial cells were programmed to override differentiation-inducing signals. Moreover, they were committed to a phenotype of increased oxidoreductase activity, fatty acid β-oxidation, tricarboxylic acid cycle, and the respiratory electron transport chain, in addition to overexpression of genes facilitating cell cycle progression and multidrug resistance.

To determine whether the biological response to BPA described above in nonmalignant breast epithelial cells of patients with cancer was reflected within pathologically identified carcinoma

[3] http://www.broad.mit.edu/gsea/msigdb

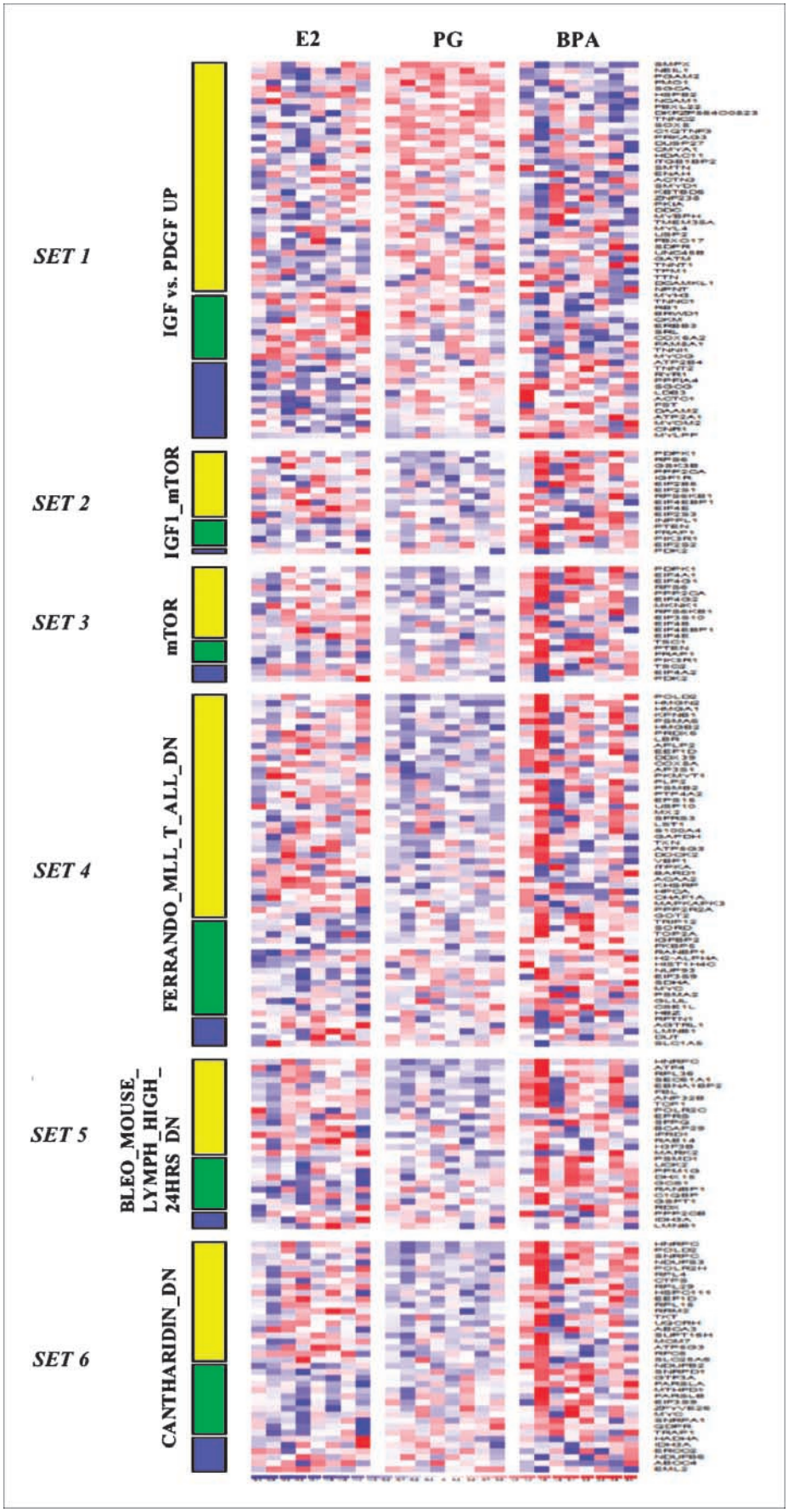

Figure 2. Derivation of the CPRP using nonmalignant epithelial-fibroblast cocultures. Heat map of response to E2, PG, and BPA in breast epithelial cells of women at high risk. *Columns under each treatment,* independent cases; *rows,* genes. Six gene sets identified by gene set analysis display similarities between E2- and BPA-induced profiles which contrast with PG-induced effects. *Yellow side bars,* genes with similar expression patterns; *non-yellow side bars,* opposing expression patterns in E2- versus BPA-exposed cells. *Green side bars,* genes down-regulated by BPA in set 1, vice versa in sets 2 to 6. *Blue side bars,* genes down-regulated by E2 in set 1, vice versa in sets 2 to 6.

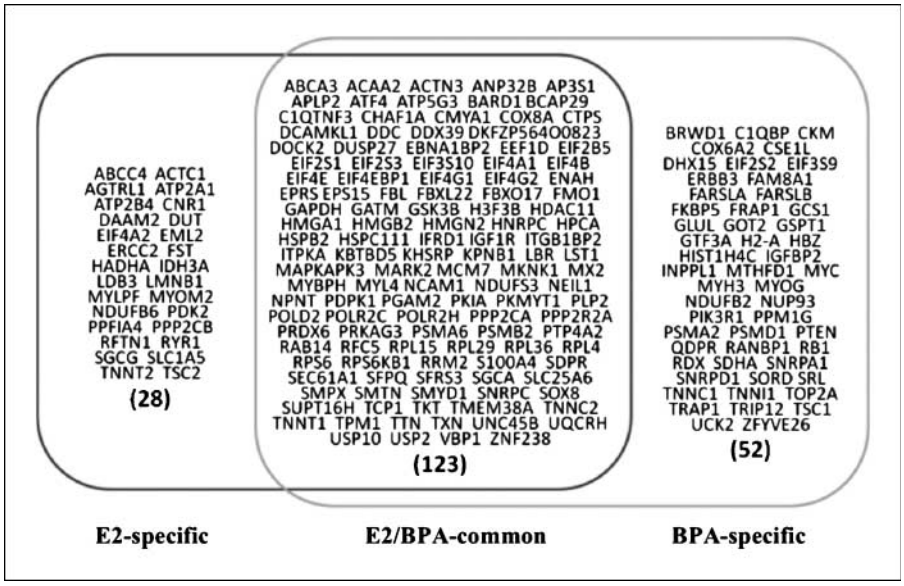

Figure 3. Distinctive E2- and BPA-associated changes in RPFNA cultures of patients at high risk of breast cancer. E2 gene profile (*left*), the BPA profile (*right*), and genes representing expression patterns common to E2 and BPA (*middle*), but reversed in the presence of PG.

from a similar cohort of patients, CPRPs associated with E2, PG, and BPA were individually applied towards class prediction in primary breast cancer data sets: GSE5460 (n = 125) and GSE6532 (n = 414; ref. 15). First, the cell culture–based classifiers were used to stratify ER status, tumor size, and tumor grade. CPRP-BPA was more prevalent in ER-negative tumors (P = 0.0055), which generally reflect the most aggressive disease subset. This profile was also displayed by tumors >2 cm in size (P = 0.0022), and higher histologic grade (grade 1 versus 3, P = 0.000002; grade 1 versus 2 versus 3, P = 0.000009; Fig. 4*A* and *B*). Histologic grade is the most consistent indicator of breast cancer aggressiveness. One-third of all breast cancers display a high-grade phenotype leading to disease-related mortality (16, 17). Clinical follow-up data (GSE6532), revealed that tumors which displayed CPRP-E2, or CPRP-BPA, conferred a significantly poor prognosis compared with those that displayed CPRP-PG (P = 0.0014 and P = 0.00057, Fig. 4*C* and *D*, respectively).

In terms of its striking association with tumor aggressiveness, it is to be noted that in contrast to global gene signatures preselected for correlation with breast cancer prognosis (18, 19), CPRP was derived from a direct functional cellular response restricted to six well-known biological pathways implicated in tumorigenesis. Another important point of distinction from other predictive signatures is that in the derivation of CPRP, major cell proliferation genes did not attain significance. This is likely due to the fact that gene expression profiles were compared here between cell cultures of equivalent growth rate. A similar observation was made in the identification of a cancer cell immortalization signature based on the comparison of finite life (but proliferating) and immortalized primary breast tumor cells (20). Thus, the association of CPRP-BPA with breast cancer aggressiveness does not merely reflect a correlation between proliferating cells *in vitro* and those in high-grade tumors. Instead, it reveals cellular alterations that may play an underlying role in the induction and maintenance of dysregulated cell proliferation in cancer, thereby influencing patient outcome. The robust similarity observed here between the phenotypes of BPA-exposed nonmalignant cell cultures and full-blown aggressive breast tumors provides a conceptual framework for the detection of a poor prognosis signature even at the earliest disease stages (18).

Unlike classical methods of evaluating endocrine-disrupting agents by *in vitro* ER ligand–binding and transactivation assays, our comprehensive genomics-based evaluation of BPA exposure in conjunction with a paired study design that minimizes the effects of confounding factors has successfully tracked early causal changes in the context of nonmalignant breast tissue. Such assays which yield data that is biologically relevant to tumor-promoting alterations could be invaluable for determining which chemicals might pose a long-term threat to human health, enabling a meaningful payoff from focused epidemiologic studies of breast cancer and the environment.

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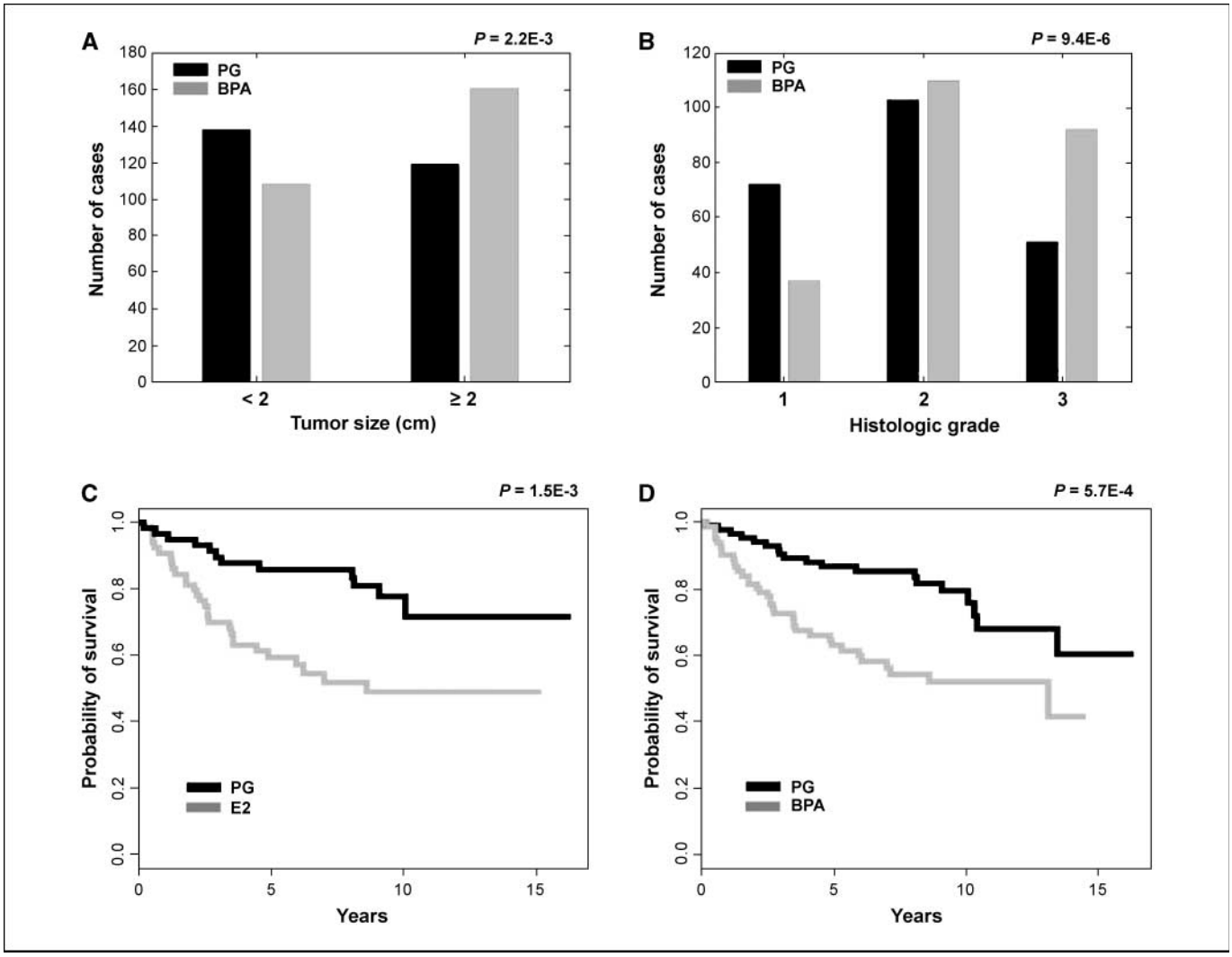

Figure 4. Application of the CPRP towards class prediction of primary breast cancer. *A* and *B,* the BPA-induced profile is significantly associated with larger tumor size and higher histologic grade in comparison with the PG-induced expression profile. *C* and *D,* Kaplan-Meier survival curves for subgroups with high correlation to profiles induced by E2, PG, and BPA. Compared with CPRP-PG, both CPRP-E2 and CPRP-BPA independently predict a worse prognosis for patients with breast cancer.

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